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IDENTIFICATION OF POSSIBLE REGULATORY MOLECULES FOR CHONDROCYTE ANABOLISM IN OSTEOARTHRITIC CARTILAGE

N. Fukui[1], Y. Ikeda[1], T. Ohnuki[1], N. Tanaka[1], S. Ishida[1], S. Yamane[1], H. Mitomi[1], A. Hikita[1], S. Yamamoto[2], Y. Katsuragawa[3], R. Suzuki[1]
[1]Clinical Research Center, National Hospital Organization Sagamihara Hospital, Sagamihara, Kanagawa Pref, Japan; [2]Tokyo Metropolitan Geriatric Hospital, Itabashi, Tokyo, Japan; [3]International Medical Center of Japan, Shinjyuku, Tokyo, Japan

Purpose: In OA cartilage, the metabolic activity of chondrocytes is altered dramatically by site, but the mechanism(s) for the changes is not known well. The purpose of this study is to determine possible regulatory molecules for chondrocyte anabolism in OA.
Methods: In order to determine the candidate molecules which may regulate chondrocyte anabolism in OA, cartilages were obtained from 10 end-stage OA knees at macroscopically preserved areas and degenerated areas. RNA was extracted from the 10 pairs of cartialge samples, and the profiles of gene expression at respective areas were determined by microarray analysis (U133 Plus 2.0, Affymetrix). Linear regression analysis was performed to evaluate the relatiohship between the expression of type II collagen or aggrecan and that of the growth factors which were known to have anabolic effects on chondrocytes (TGF-βs, BMPs, IGFs, CTGF). The correlation was also investigated between the cartilage matrices and receptors and endogenous inhibitors for the growth factors. Further, the correlation was investigated between the cartilage matrices and the cartilage related SOXs (SOX5, SOX6, SOX9).
For some candidate molecules, the correlation of expression was confirmed by an analysis using real-time PCR and laser capture microdissection (LCM). For this, OA cartilages were acquired from another 10 OA knees at various sites in preserved and degenerated areas. Cryosections were prepared and divided into three cartilage zones (superficial, middle, and deep zones) by LCM (PixCell IIe, Arcturus). The expression of cartilage matrices and candidate molecules was evaluated in respective zones by real-time PCR.
Results: In the microarray analysis, a significant correlation was observed between the expression of type II collagen and aggrecan, which was confirmed by the real-time PCR analysis coupled with LCM. The result of microarray analysis revealed that the expression of type II collagen was positively correlated with that of IGF-2 and TGF-β receptors (Table 1). Of these, the significance of correlation between type II collagen and IGF-2 was confirmed by the real-time PCR analysis. Meanwhile, the expression of aggrecan was positively correlated with that of IGF-2, IGF-2 receptor, TGF-β1, and TGF-β receptors, and negatively correlated with that of IGF binding proteins and latent TGF-β binding proteins (Table 1). The real-time PCR analysis confirmed significant correlations between aggrecan and IGF-2, TGF-β1, and IGF binding proteins-3 and -7.
The expression of type II collagen and aggrecan was both significantly correlated with that of SOX9, but not with SOX5 or SOX6, which was also confirmed by the real-time PCR analysis.
Conclusions: Although the anabolic activity of chondrocytes is highly enhanced in the preserved areas in OA cartilage, it is considerably reduced at the degenerated areas, which may be responsible for the loss of cartilage matrix in OA. The result of this study suggested that the change in IGF-2 and TGF-β1 activities may account for such regional difference in chondrocyte anabolism. SOX9 could be another factor for the regional change, though the relationship between SOX9 and the above two growth factors is still left to be determined. Although further experiments are necessary, this result could be a novel clue to dissect the mechanisms for altered chondrocyte metabolism in OA.

Table 1. Correlation of expression between cartilage matrices and growth factors, growth factor related molecules

Cartilage matrices	Growth factors and related molecules			
	Positively correlated		Negatively correlated	
Type II collagen	IGF-2*	(r = 0.59, $P < 0.01$)		
	TGF-β RI	(r = 0.45, $P < 0.05$)		
	TGF-β RII	(r = 0.47, $P < 0.05$)		
Aggrecan	IGF-2*	(r = 0.78, P< 0.01)	IGFBP-1	(r = -0.60, $P < 0.01$)
	IGF-2 R	(r = 0.78, $P < 0.01$)	IGFBP-3*	(r = -0.65, $P < 0.01$)
			IGFBP-4	(r = -0.64, $P < 0.01$)
			IGFBP-5	(r = -0.68, $P < 0.01$)
			IGFBP-6	(r = -0.64, $P < 0.01$)
			IGFBP-7*	(r = -0.62, $P < 0.01$)
	TGF-β1*	(r = 0.70, $P < 0.01$)	LTBP-I	(r = -0.47, $P < 0.05$)
	TGF-β RII	(r = 0.66, $P < 0.01$)	LTBP-2	(r = -0.46, $P < 0.05$)
	TGF-β RIII	(r = 0.79, $P < 0.01$)	LTBP-3	(r = -0.46, $P < 0.05$)

*significance of correlarion was confirmed by real-time PCR analysis. IGFBP, IGF binding protein; LTBP, latent TGF-β binding protein.

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INORGANIC PYROPHOSPHATE GENERATION BY TUMOR GROWTH FACTOR-BETA1 IS MAINLY DEPENDENT ON ANK INDUCTION BY RAS/RAF-1/EXTRACELLULAR REGULATED KINASE PATHWAYS IN CHONDROCYTES

F. Cailotto, S. Sebillaud, D. Moulin, J-Y. Jouzeau, **P. Netter**, A. Bianchi
Laboratoire de Physiopathologie et Pharmacologie Articulaires, Vandoeuvre les Nancy, France

Purpose: Sporadic chondrocalcinosis is a common human disorder characterized by the deposition of calcium-containing crystals, mostly calcium pyrophosphate dihydrate (CPPD), within articular cartilage. ANK is a multipass transmembrane protein transporter, thought to play a role in the export of intracellular inorganic pyrophosphate (iPPi), and so to be implicated in chondrocalcinosis. Two others proteins play a major role in the metabolism of inorganic pyrophosphate: PC-1, that generates extracellular inorganic pyrophosphate (ePPi) from nucleotides triphosphates, and TNAP (Tissue Non specific Alkaline Phosphatase), that cleaves one molecule of ePPi into two molecules of extracellular inorganic phosphate (ePi). Transforming growth factor-β1 (TGF-β1) was shown to enhance ePPi production by articular chondrocytes, markedly in old patients than in young patients, this was closely related to the occurrence of sporadic chondrocalcinosis. Therefore, we investigated the contribution of ANK to the production of extracellular ePPi by chondrocytes and the signaling pathways involved in the regulation of Ank gene by TGF-β1.
Methods: Chondrocytes were exposed to 10 ng/ml of TGF-β1, and Ank expression was measured by quantitative PCR and Western blot analyses. ePPi and ePi were quantified concomitantly by a fluorescence assay in cell supernatants. SiRNA technology was used to define the respective role of Ank and PC-1 in TGF-β1-induced ePPi generation. Finally, selective inhibitors and dominant negative/constitutively active overexpression plasmids strategy were used to explore the contribution of Ras, Raf-1, MAPKs (ERK 1/2 and p38 MAPK), Smad, PKA and PKC pathways to Ank induction by TGF-β1.
Results: TGF-β1 strongly increased Ank expression, maximally by 4.5 fold at the mRNA and by 4-fold at the protein level. PC-1 mRNA and protein levels were induced with the same intensity, but in a more delayed fashion, whereas TNAP was repressed by 2-fold under TGF-β1 stimulation. Moreover, ePPi production was induced 5-fold after 24 h TGF-β1 exposure, whereas ePi levels were not affected under the same conditions. Then, we

demonstrated that siRNA for Ank reduced 4-fold more than siRNA for PC-1 the inducing effect of TGF-β1 on ePPi levels. We showed that neither SB203580 (a p38 MAPK inhibitor), nor RcAMP (a PKA inhibitor) were able to modulate Ank induction, but that PD98059 (a MEK 1 inhibitor) was strongly active, ensuring a 50% inhibition of TGF-β-1-induced Ank expression. On the other hand, the effect of PKC inhibitors depended on their selectivity: PKCδ inhibitor (rottlerin) was weakly effective compared to Ca2+-dependent PKC inhibitor (Gö6976), that diminished Ank expression by 60%, suggesting the involvement of calcium-dependent PKC in the regulation of Ank gene by TGF-β1. Finally, we demonstrated with plasmids transfection the involvement of Ras, Raf-1 and confirmed the contribution of ERK in Ank mRNA regulation. However, overexpression of Smad 7 did not affect Ank expression, showing that Smad-dependent signaling events downstream to TGF-β1 were not implicated.
Conclusions: These results indicate that TGF-β1 mediates an increase in ePPi concentration, mainly by induction of Ank expression, which depends on activation of Ras, Raf-1, ERK and Ca2+-dependent PKC pathways in chondrocyte. Our results underline the interest to modulate Ras/Raf-1/ERK or PKC signaling pathways in chondrocytes, as it could open insights for the treatment of sporadic chondrocalcinosis.

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MOLECULAR INTERACTION BETWEEN BCL-XL AND BNIP3 DETERMINES THE CELL FATE OF HYPERTROPHIC CHONDROCYTES

Y. Oshima[1], T. Akiyama[1], M. Iwasawa[1], Y. Nagase[1], K. Nakamura[1], L. Hennighausen[2], H. Kawaguchi[1], S. Tanaka[1]
[1]The University of Tokyo, Tokyo, Japan; [2]National Institutes of Health, Bethesda, MD

Purpose: It has been reported that the early stage of osteoarthritis (OA) is characterized by hypertrophic differentiation and apoptosis of chondrocytes, which mimics the developmental process of growth plate chondrocytes. The level of inorganic phosphate (Pi) elevates at the site of cartilage mineralization, and previous studies have proposed that Pi entry into hypertrophic chondrocytes may act as an apoptogen. The Bcl-2 family proteins, which consist of pro- and anti-apoptotic members, are major regulators of mitochondria-initiated apoptosis. In this study, we investigated how they determine the cell fate of chondrocytes.
Methods: We developed an *in vitro* system to analyze the mechanism of hypertrophic differentiation and apoptosis of chondrocytes using chondrogenic ATDC5 cells. Using this culture system, the expression levels of pro- and anti-apoptotic Bcl-2 members were analyzed by real time RT-PCR or western blotting, and their roles in chondrocyte apoptosis by gene overexpression and gene silencing. Association between anti-apoptotic member Bcl-xL and pro-apoptotic member Bnip3 was examined by immunoprecipitation, and their expression patterns in growth plate chondrocytes were investigated by immunohistological staining. Finally, we generated chondrocyte-specific knockout mice of anti-apoptotic Bcl-2 family member protein Bcl-xL using the Cre-loxP recombination system, and examined its roles *in vivo*.
Results: When differentiated ATDC5 cells were treated with Pi, they mineralized the surrounding matrix and underwent rapid apoptosis as evidenced by nuclear condensation, Caspase-3 & 7 activation, and Lamin proteolysis. In this culture system, among 15 pro-apoptotic Bcl-2 family members, 7 molecules increased their expression levels during the course of hypertrophic differentiation, and two in response to Pi stimulation. Of these, gene silencing of a proapoptotic BH3-only molecule *bnip3* by RNA interference significantly suppressed Pi-induced apoptosis. Conversely, among anti-apoptotic members examined, overexpression of *bcl-xL* suppressed, and its knockdown promoted apoptosis. The susceptibility to apoptosis by *bcl-xL* knockdown was partially restored by simultaneous silencing of *bnip3*. Pi treatment markedly upregulated the protein levels of Bnip3 without affecting Bcl-xL levels. Bnip3 was associated with Bcl-xL and attenuated its anti-apoptotic effect in chondrocytes. Immunohistological examination of murine growth plates revealed that Bcl-xL expressed uniformly in the growth plate chondrocytes, whereas Bnip3 expression was exclusively localized in the hypertrophic chondrocytes. Finally, we generated chondrocyte-specific *bcl-xL* knockout mice using the Cre-loxP recombination system, and provided evidence that the hypertrophic chondrocyte layer was markedly shortened in those mice owing to massive chondrocyte apoptosis and that the mice exhibited dwarfism as a result. Furthermore, Bnip3 expression was decreased in hypertrophic chondrocytes in mice fed with a low phosphate diet, and the abnormalities in the growth plate were almost completely rescued.
Conclusions: Increase in Pi levels in hypertrophic zone causes upregulation of Bnip3 in chondrocytes, which binds to Bcl-xL and consequently impairs its anti-apoptotic effect, and finally causes apoptosis of the cells. Our observations will open a new therapeutic approach for OA by modulating the function of Bcl-xL/Bnip3 axis and consequently regulating chondrocyte apoptosis.

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INTERLEUKIN 6 (IL-6) SIGNALING MODULATES ANABOLIC AND CATABOLIC PATHWAYS IN HUMAN ARTICULAR CHONDROCTYES

M. Lu, S. Mandala, **J. Menetski**
Merck Research Laboratories, Rahway, NJ

Purpose: Interleukin 6 (IL-6) production is elevated in affected joints of osteoarthritis (OA) patients. The central role played by IL-6 in joint inflammation and destruction has suggested therapies targeting IL-6 for treatments of arthritic diseases. However, increased severity of spontaneous cartilage damage in IL6-/- mice suggests a protective role by IL-6 in joint homeostasis. The aim of this study was to examine the effects of blocking IL-6 signaling on both catabolic and anabolic gene regulation in human primary chondrocytes (HACs) by small interfering RNA (siRNA) technology and antibody treatment.
Methods: Small interfering RNA-mediated gene-specific knockdown of IL-6 signaling components (IL-6 or IL-6R) was applied to achieve blockade of IL-6 signaling in human primary chondrocytes (HACs) treated with catabolic (IL-1β) or anabolic (TGF-β or IGF-1+OP-1) stimuli. To confirm the siRNA effects in chondrocytes, a neutralizing anti-IL-6 antibody was also studied for effects in response to IL-1β treatment. Expression of the mRNA for chondrocytic genes was determined by quantitative real-time PCR (Taqman). The protein level of IL-6 and MMP-13 were measured using immunochemical based assays.
Results: Transfection of IL-6 and IL-6R siRNA into chondrocytes resulted in significant gene specific knockdown of IL-6 and IL-6R mRNA, respectively. In addition, knockdown of IL-6 led to a drastic reduction in IL-1β-induced expression of IL-6 mRNA and protein. Decreased IL-6 expression also led to a marked reduction in IL-1β-induced expression of pro-inflammatory cytokine TNF-α, and cartilage degrading enzymes such as MMP-13 and ADAMTS-4. The inhibitory effects by IL-6 siRNA were specific to IL-6 signaling, as similar effects were exhibited by both IL-6 neutralizing antibody and IL-6R siRNA. Surprisingly, IL-6 appears to modulate gene expression in chondroctyes under anabolic stimulation. We found that the expression of several essential ECM and cartilage components, in particular collagen II (Col2a), was not induced by TGF-β or IGF-1/OP-1 in cells transfected with IL-6 siRNA.